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Pollen fertility and the role of ROS and Ca signaling in heat stress tolerance

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Project award year: 2013
Three year research project

Abstract

The long-term goal of this research is to understand how pollen cope with stress, and identify genes that can be manipulated in crop plants to improve reproductive success during heat stress.

The specific aims were to: **1) Compare heat stress dependent changes in gene expression between wild type pollen, and mutants in which pollen are heat sensitive (*cngc16*) or heat tolerant (*apx2-1*). 2) Compare *cngc16* and *apx2* mutants for differences in heat-stress triggered changes in ROS, cNMP, and Ca^{2+} transients. 3) Expand a mutant screen for pollen with increased or decreased thermo-tolerance.** These aims were designed to provide novel and fundamental advances to our understanding of stress tolerance in pollen reproductive development, and enable research aimed at improving crop plants to be more productive under conditions of heat stress**.**

Background: Each year crop yields are severely impacted by a variety of stress conditions, including heat, cold, drought, hypoxia, and salt. Reproductive development in flowering plants is highly sensitive to hot or cold temperatures, with even a single hot day or cold night sometimes being fatal to reproductive success. In many plants, pollen tube development and fertilization is often the weakest link. Current speculation about global climate change is that most agricultural regions will experience more extreme environmental fluctuations. With the human food supply largely dependent on seeds, it is critical that we consider ways to improve stress tolerance during fertilization.

The heat stress response (HSR) has been intensively studied in vegetative tissues, but is poorly understood during reproductive development. A general paradigm is that HS is accompanied by increased production of reactive oxygen species (ROS) and induction of ROS-scavenging enzymes to protect cells from excess oxidative damage. The activation of the HSR has been linked to cytosolic Ca^{2+} signals, and transcriptional and translational responses, including the increased expression of heat shock proteins (HSPs) and antioxidative pathways.

The focus of the proposed research was on two mutations, which have been discovered in a collaboration between the Harper and Miller labs, that either increase or decrease reproductive stress tolerance in a model plant, *Arabidopsis thaliana* (i.e., *cngc16--cyclic nucleotide gated channel 16, apx2-1--ascorbate peroxidase 2,*).

Major conclusions, solutions, achievements. Using RNA-seq technology, the expression profiles of *cngc16* and *apx2* pollen grains were independently compared to wild type under favourable conditions and following HS. In comparison to a wild type HSR, there were 2,776 differences in the transcriptome response in cngc16 pollen, consistent with a model in which this heat-sensitive mutant fails to enact or maintain a normal wild-type HSR. In a comparison with apx2 pollen, there were 900 differences in the HSR. Some portion of these 900 differences might contribute to an improved HSR in *apx2* pollen. Twenty-seven and 42 transcription factor changes, in *cngc16* and *apx2-1*, respectively,

were identified that could provide unique contributions to a pollen HSR. While we found that the functional HS-dependent reprogramming of the pollen transcriptome requires specific activity of CNGC16, we identified in *apx2* specific activation of flavonol-biosynthesis pathway and auxin signalling that support a role in pollen thermotolerance. Results from this study have identified metabolic pathways and candidate genes of potential use in improving HS tolerance in pollen.

Additionally, we developed new FACS-based methodology that can quantify the stress response for individual pollen in a high-throughput fashion. This technology is being adapted for biological screening of crop plant's pollen to identify novel thermotolerance traits.

Implications, both scientific and agricultural. This study has provided a reference data on the pollen HSR from a model plant, and supports a model that the HSR in pollen has many differences compared to vegetative cells. This provides an important foundation for understanding and improving the pollen HSR, and therefor contributes to the long-term goal of improving productivity in crop plants subjected to temperature stress conditions. A specific hypothesis that has emerged from this study is that pollen thermotolerance can be improved by increasing flavonol accumulation before or during a stress response. Efforts to test this hypothesis have been initiated, and if successful have the potential for application with major seed crops such as maize and rice.

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
Review Article	0	1	0
Reviewed	1	1	0
Submitted	0	1	0

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
Ph.D. Student	Ishka	Maryam	UNR	USA
Ph.D. Student	Weigand	Chyrstle	UNR	USA
Ph.D. Student	Luria	Gilad	BIU	Israel
M.Sc. Student	Malihi	Shimrit	BIU	Israel
M.Sc. Student	Shimanovsky	Vlada	BIU	Israel

Contribution of the collaboration

The responsibility for the three objectives was equally divided between the Harper and Miller groups and each side was involved in all aspects of the project in order to achieve a synergistic and fruitful outcome.

The on-going dialog between the two research groups has been valuable for promoting the original objectives and for developing new hypothesis that originated from the results obtained. JH and GM have been regularly discussing the progress of the project by mail or skype video calls. There has been a fertilizing exchange of ideas and biological material between the labs.

This mode of cooperation allowed us to be well coordinated and decide on the best action to take during the study that will best serve the end goals of the project. For example, in aim 1, two independent RNAseq comparisons were conducted by each group; one for the *cngc16* and the other for *apx2-1* mutant. However to obtain maximum uniformity and basis for further comparison between the two experiments, the RNAseq pipeline that was used was the same (the same sequencing platform, Ilumina Hiseq^{TM}2000 by the same service provider, BGI), and the bioinformatics data analyses was done at UNR). During the bioinformatics analysis of the samples from the UNR pollen experiment, which were processed first, we recognize a need to include leaf samples that will serve as a reference for the pollen experiment. Thus, we included leaf samples together with the pollen samples in the BIU experiment for RNAseq analysis. This change in the original plan, which was crucial for our success in identifying pollen-specific HSR genes and pathways, is the outcome of our close cooperation.

In addition, both partners have extended their cooperation by joining pollen groups that are also interested in the response of pollen to abiotic stress; Nurit Firon and Michal Lieberman-Lazarovich (The Volcani Center, ARO, Israel) and Ann Loraine (University of North Carolina).

In the summer of 2015, Drs. Harper and Miller met in the Minneapolis MN during the pollen RCN meeting and later in the ASPB meeting. Both have presented progress made in their research during the two meeting and elaborately discussed current and future plans to broaden the collaboration between the two labs. On March 2017, Dr. Harper visited in Israel. During his visit JH presented his work in seminars at Bar Ilan University and at Tel Aviv University. In addition, joint discussions were held, which included students from the Miller lab and researchers from ARO Volcani center.

Achievements.

Significance of main scientific achievements or innovations (detailed for each of the three specific aims).

Aim 1) Compare heat stress dependent changes in gene expression between wild type pollen, and mutants in which pollen are heat sensitive (*cngc16*) or heat tolerant (*apx2*).

UNR led: Plants harboring a *cncg16* knockout are nearly sterile under conditions of hot days and cold nights. To understand the underlying cause, RNA-Seq was used to compare the pollen transcriptomes of wild type (WT) and *cngc16* under normal and heat stress (HS) conditions. Our results define an extensive HS (heat-stress)-dependent reprogramming of approximately 15% of the WT pollen transcriptome (≥ 2-fold changes with adjusted *p*-value ≤ 0.01). In contrast to WT, *cngc16* pollen showed 1.9-fold more HS-dependent changes (3936). Our results support a model in which a functional HS-dependent reprogramming of the pollen transcriptome requires a specific calcium-permeable *Cyclic Nucleotide-Gated cation Channel, CNGC16.* A manuscript presenting this part of the study was recently submitted to 'BMC Genomics' journal.

BIU led: Plants harboring an *apx2-1* mutation have pollen that appear more HS tolerant. To understand the underlying cause, RNA-Seq was used to compare the pollen transcriptomes of wild type (WT) and *apx2-1* pollen with and without a HS. Additionally, the expression profile of leaf was performed to help identify pollen-specific HS-dependent changes. Our results showed that 89% of the HS-dependent changes in pollen were different from those observed in leaves. This supports a hypothesis that the pollen and leaves have significant difference in the reprograming of their transcriptomes in response to a HS. Among the changes that were specific to *apx2-1* pollen, we identified significant changes in mRNAs associated with 1) enzymes involved in flavonol biosynthesis and 2) with responses to the plant hormone auxin. Because the literature provides evidence that flavonols and auxin can cross-regulate one another, we hypothesize that changes in these pathways might function in a synergistic fashion to enhance thermotolerance of the *apx2-1* pollen. Transgenic plants that over-produce

flavonols in pollen and over-express aux/IAA transcription factors are being generated and will be tested for HS tolerance.

Aim 2) Compare *cngc16* and *apx2* mutants for differences in heat-stress triggered changes in ROS, cNMP, and Ca2+ transients.

UNR led: Reporters for detecting Ca^{2+}, cGMP signals were stably transformed into *cngc16* and wild type plants (YC3.6 cameleon, FlincG delta reporter for cGMP, EPAC reporter for cAMP). In addition, we engineered plants with two genes to specifically degrade cAMP and cGMP (Phosphodiesterase 4 and 5 from mouse, PDE4 and PDE5). To our surprise, we did not observe the expected near-sterile phenotype for pollen in which the cNMPs were presumably being degraded. A near-sterile phenotype was expected since CNGC18 (cyclic nucleotide gated channel 18) has been shown to be essential for pollen fertility. Alternative constructs have been designed and are being tested to determine if pollen fitness can be negatively impacted by targeting PDEs to the plasma membrane, or through creating constitutively active versions of PDE5. The current absence of a PDE effect on pollen raises a question about whether the plant CNGC channels have evolved to respond to different regulatory molecules. Thus, this result has delayed our plans to try and image changes in the concentrations of cNMPs.

BIU led. We developed a novel cytometric-based method to quantify changes in reactive oxygen species (ROS) and redox changes that will replace the time consuming and inaccurate microscope-based approach. Using a fluorescence-activated cell-sorting (FACS) device we have been able to record dynamic changes in ROS accumulation in a large population of Arabidopsis and tomato pollen grains. Using this technique wild type and *apx2-1* pollen were compared, however no significant differences in ROS levels have yet been detected. This suggests that any difference in ROS dynamics between wild type and *apx2-1* are either small, very transient, or highly localized.

Nevertheless, our FACS method has successfully been used as an assay to rapidly evaluate pollen viability. This method has potential applications in testing or selecting for pollen traits of agricultural merit (such as heat stress tolerance). A manuscript

describing the new method is in preparation.

Aim 3) Expand a mutant screen for pollen with increased or decreased thermo-tolerance.

UNR led. Each year, more than 10 genetic knockouts or over-expression lines were screened for changes in reproductive fitness under heat stress conditions. Several candidate genes of interest have been identified, including a putative acetyl-transferase that potential modifies the pollen cell wall.

BIU led. We have completed reciprocal crosses and the selfing crosses experiments with two independent alleles of *apx2*, *apx2-1* and *apx2-2*. Experiments conducted with *apx2-1* clearly prove that this mutant allele improves reproductive success under high temperature by rendering the male gametophyte more tolerant to HS. Surprisingly, the experiments with the second allele, *apx2-2* showed that it is just as sensitive as the WT to HS. Thus, evidence indicates that *apx2-1* and *apx2-2* have different phenotypes.

Agricultural and/or economic impacts of the research findings

Temperature stress is a major contributor to crop loss around the world, with pollen infertility being one of the most important underlying causes. Results here using a model plant system have identified 1000s of specific HS-dependent changes in the pollen transcriptome, and showed that the pollen HSR is significantly different than what occurs in vegetative tissues. Our results suggest a strategy to increase pollen heat stress tolerance by manipulating several genes related to flavonol biogenesis. A pilot study to test this strategy in tomato has been initiated. If successful, this strategy has the potential to be applied to corn, rice, and wheat.

Changes to the original research plan.

As we have been testing ROS accumulation in pollen grains and tubes of WT and *apx2* under favorable conditions and during HS, using microscopy and ROS-specific fluorescent dyes, we became aware of the limitation of this approach for obtain reliable and accurate results for a large number of events (i.e. pollen grains).

Therefor, we have been diverting a significant proportion of our efforts at BIU to develop a flow cytometry-based method using a FACS device to evaluate ROS level s using the ROS probe H2DCF-DA.

This approach proved accurate and robust and were further extend to evaluate pollen viability and other metabolic activities. This method has been tested on pollen from wild type, apx2-1 and other mutants and transgenic plants overexpressing gene of interest that were identified in the project. This method has been tested not only on Arabidopsis, but also on tomato and tobacco pollen.

We are now in the final stages of writing the manuscript and intending to submit it during fall of 2017. Due to this deviation form our original plan, other experiments that have been pushed aside, will now benefit from this new method and are expected to provide significant results. These experiments also include testing redox changes using the redox sensor roGFP in pollen of the wild type and apx2 mutant.

In addition, the discovery of the specific activation of flavonol synthesis and auxin signaling in the apx2-1 mutant during heat stress, have been guided us to focus on this direction and continue to investigate the function of these two pathways in pollen thermotolerance. Thus much of the 3rd year at BIU was dedicated for experiment using flavonol deficient and over accumulating mutants as well as auxin biosynthesis and signaling mutants. This decision proved itself, as significant progress has been made tying both auxin and flavonol accumulation to pollen fertility. We are now trying to better understand the relationship between these two pathways in pollen on a mechanistic level.

We expect to have a manuscript ready during the spring of 2018.

Our findings are highly important and we are already testing, using transgenic approaches, whether increasing the affect of flavonols and auxin in pollen can increase reproductive success during heat stress

Publications for Project IS-4652-13 R

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Published	Reviewed	*Ann E. Loraine, Ivory Clabaugh Blakley, Sridharan Jagadeesan, Jeff Harper, Gad Miller, and Nurit Firon*	Analysis and Visualization of RNA-Seq Expression Data Using RStudio, Bioconductor, and Integrated Genome Browser	***Methods in Molecular Biology***	1284 : 481-501 2015	Joint
Published	Review Article	*Won-Gyu Choi1, Gad Miller, Ian Wallace, Jeffrey Harper, Ron Mittler and Simon Gilroy*	Orchestrating rapid long-distance signaling in plants with Ca2+, ROS and electrical signals	***Plant Journal***	90 : 698-707 2017	Joint
Submitted	Reviewed	*Maryam Rahmati Ishka, Elizabeth Brown, Chrystle Weigand, Richard Tillett, Karen Schlauch, Gad Miller, Jeffrey F. Harper*	A comparison of heat-stress transcriptome changes between wild-type Arabidopsis pollen and a heat-sensitive mutant harboring a knockout of cyclic nucleotide-gated cation channel 16 (cngc16)	***BMC Genomics***	: 2017	Joint
Published	Reviewed	*Changming Chen, Shir Twito & Gad Miller*	New cross talk between ROS, ABA and auxin controlling seed maturation and germination unraveled in APX6 deficient Arabidopsis seeds	***Plant Signaling & Behavior***	9 : e976489 2015	IS only